(54) Expression vector for human topoisomerase I

Expressionsvektor für menschliche Topoisomerase I

Vecteur d'expression pour humain topisomerase I

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Description

[0001] DNA topoisomerases are ubiquitous enzymes which catalyze the breakage and rejoining of the DNA phosphodiester backbone (J. C. Wang, Annu. Rev. Biochem., 54, pages 665-697, 1985; P.-H. Vosberg, Curr. Top. Microbiol. Immunol, 114, pages 19-102, 1985). These reactions, together with an intervening strand passage event, allow this class of enzymes to alter DNA topology. Intermediates in the strand passage reaction may involve either single or double stranded breaks allowing the classification of topoisomerases into type I and type II enzymes respectively. In eukaryotic cells, including human cells, a single type I enzyme has been described (J. C. Wang, Biochem. Biophys. Acta., 909, pages 1-9, 1987). The bacteria E.coli is known to possess two type I enzymes. One, encoded by the topA gene, is responsible for the major DNA relaxing activity of the cell and is essential in an otherwise wild type E.coli (G. J. Pruss et al., Cell, 31, pages 35-42, 1982; S. DiNardo et al., Cell, 32, pages 43-51, 1982). The second E.coli enzyme, topoisomerase III, is a minor activity encoded by the topB gene which appear to be completely dispensable (H. Hiasa et al., J. Biol. Chem., 269, pages 2093-2099, 1994).

[0002] Although the E.coli and human topoisomerase I both catalyze the relaxation of negatively supercoiled DNA, they differ in the details of the reactions they catalyze and share no amino acid sequence homology. The human topoisomerase I ("hTOPI") shows a preference for binding double stranded DNA and proceeds by making a single covalent 3'-phosphodiester intermediate to a tyrosine of the enzyme (M. D. Been et al., J. Mol. Biol. 180, pages 515-531, 1984; W. Baase et al., Biochemistry, 13, pages 4299-4303, 1974; J. J. Champoux, Proc. Natl. Acad. Sci. USA, 74, pages 3800-3804, 1977). The E. coli enzyme ("eTOPI") demonstrates a preference for binding at the junction of double and single stranded regions and proceeds using a single 5'-phosphodiester intermediate (J. C. Wang, J. Mol. Biol., 55, pages 532-533, 1971; K. Kirkegaard et al., J. Mol. Biol., 185, pages 625-637, 1985; R. E. Depew et al., J. Biol. Chem., 253, pages 511-518, 1978). The eTOPI enzyme is very efficient at relaxing highly negatively supercoiled DNA and shows progressively decreasing activity as the substrate DNA becomes more relaxed (J. C. Wang, J. Mol. Biol., 55, pages 531-533, 1971; K. Kirkegaard et al., Proc. Natl. Acad. Sci. USA, 184, pages 625-637, 1985). The hTOPI enzyme relaxes both negatively and positively supercoiled DNA to completion (W. Baase et al., Biochemistry, 13, pages 4299-4303, 1974). The hTOPI enzyme is the target of the antitumor drug camptothecin (CMPT) which traps the covalent phosphotyrosine intermediate of the strand passage reaction (Y.-H. Hsiang, et al., J. Biol. Chem., 260, pages 14873-14878, 1985.). E.coli eTOPI enzyme (K. Drlica et al., Biochemistry, 27, pages 2253-2259) is resistant to CMPT.

[0003] It previously has been shown that plasmid borne copies of both the yeast (M.-A. Bjornsti et al., Proc. Natl. Acad. Sci. USA, 84, pages 8971-8975, 1987) and human topoisomerase I (M.-A. Bjornsti et al., Cancer Research, 49, pages 6318-6323, 1989) coding sequences possess the capacity to complement a conditional defect in the bacterial topA gene. Subsequent to this study the same group of researchers have pursued the development of yeast model systems for the study of the eukaryotic topoisomerase I (J. Nitiss et al., Proc. Natl. Acad. Sci. USA, 85, pages 7501-7505, 1988; M.-A. Bjornsti et al., Cancer Research, 49, pages 6318-6323, 1989). Heretofore, no E. coli system useful as a model for the expression of hTOPI has been reported. This present invention concerns an E.coli system for the controlled functional expression of hTOPI.

[0004] In one aspect the present invention concerns an expression vector comprising:

(a) hTOPI DNA,
(b) a promoter, capable of directing expression of hTOPI, and
(c) lac Iq DNA which encodes a repressor for the promoter of (b).

[0005] In another aspect the present invention concerns
a prokaryotic host cell containing an expression vector comprising:

(a) hTOPI DNA,
(b) a promoter, capable of directing expression of hTOPI, and
(c) lac Iq DNA which encodes a repressor for the promoter of (b).

[0006] In another aspect the present invention concerns
a method for detecting a poison of human topoisomerase comprising:

(A) culturing a prokaryotic host cell containing the imp mutation and an expression vector containing hTOPI DNA under conditions sufficient to express human topoisomerase, in the presence of a sample which may contain a poison of human topoisomerase, and
(B) monitoring DNA damage of the host cell.

[0007] By the term "poison" is meant a compound which traps the hTOPI cleavable-complex intermediate. This phe-

nomenon will be explained in more detail hereinafter.

Figure 1.   Construction of a low-copy number regulated hTOPI expressing clone. The plasmid pMS421 was partially digested with EcoRI and the unit sized linear fragments were gel purified. Such linear pMS421 was ligated with EcoRI cut ptac-hTOP, and spectmomycin (SPC) resistant transformants (50 µgram/ml SPC, Sigma) were selected. Several SPC resistant and ampicillin (AP) sensitive transformants were analysed using a battery of mutiple restriction digests. The desired transformant, pMStop1, was identified among these selected transformants. All restiction digests, ligations, gel electrophoresis, plasmid perparations and fragment purification were done according to the vendors recommendations (Promega) and standard procedures (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Figures 2A and 2B.   Camptothecin ("CMPT") can induce sulA-lac when hTOPI is expressed. The strain RFM2055 (imp4312 sulA-lac / pMStop1) was grown overnight in LB liquid media (T. J. Silhavy et al., Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984) with 25 µgram/ml SPC, diluted 1:100 the next morning into LB without SPC and then grown into midlog phase as an inoculum in the experiment. This culture (OD_{650} = 0.6) was diluted 1:100 into fresh LB liquid with (1mM) and without (IPTG). 190 µl aliquots (+/- IPTG) were added to duplicate 10 µl samples of drug (in 100% dimethylsulfoxide ("DMSO") distributed in the wells of a microtiter plate. The microtiter plate is incubated overnight at 30°C without shaking. Following this growth and drug exposure, final cell OD_{650} values are determined, the cells are permeablized with $CHCl_3$, and assayed for β-galactosidase activity essentially as described by R. Menzel, Anal. Biochem., 181, pages 40-50, 1989 with the following exception: the substrate chlorophenol red (CPRG) was substituted for o-nitrophenol-β-galactoside ("ONPG") and the assay's progress was monitored by following the change in OD_{595}. Final activities were calculated from a kinetic assay (with nine time points) and expressed as ΔOD_{595}/min. The upper graph (Figure 2A) shows the induction of β-galactosidase activity by the drug CMPT at the concentrations given in the figure. The lower graph (Figure 2B) shows the induction of β-galactosidase activity by the DNA gyrase inhibitor, nalidixic acid, at the concentrations given in the figure.

     The choice of CPRG allows the rapid determination of β-galactosidase activity in small samples with low levels of activity (D. C. Eustice et al., BioTechniques, 11, pages 739-742, 1991. Assays performed with ONPG give strictly analogous results. The conclusion made from the data shown in this figure can also be reached by using solid agar plates with X-gal in the agar to indicate β-galactosidase activity.

     Strain construction. The E. coli strain GR4413 (sulA-lac; O. Huisman et al., Nature, 290, pages 797-799, 1981) had the imp4312 mutation introduced by transduction as described in the legend to Table 2 (see Example 3) and was then transformed with the plasmid pMStop1 (LB-SPC at 25 µgram/ml) to produce strain 2055A. E. coli strain 2055A was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A., on December 13, 1994, under the provisions of the Budapest Treaty, and has the accession number ATCC 69736.

[0008] The present invention concerns an expression vector containing hTOP1 DNA, i.e., DNA coding for part or all of human topoisomerase I (hTOP1). Preferably, the hTOP1 DNA is cDNA.

[0009] The expression vectors of the invention comprise regulatory DNA sequences, such as the promoter and repressor, operatively linked to the DNA sequence coding for all or part of hTOPI. Other regulatory sequences optionally present are also operatively linked to the DNA sequence coding for all or part of hTOP1. As used in this context, the term "operatively linked" means that the regulatory DNA sequences are capable of directing the replication and/or the expression of the DNA sequence coding for all or part of hTOPI.

[0010] Expression vectors of utility in the present invention are often in the form of "plasmids", which refer to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

[0011] Expression vectors useful in the present invention typically contain an origin of replication, a promoter located in front of (i.e., upstream of) the DNA sequence and followed by the DNA sequence coding for all or part of hTOPI transcription, termination sequences and the remaining vector. The expression vector of the invention also preferably contains a marking sequence(s) which is capable of providing phenotypic selection in transformed host cells (i.e., a

selectable marker). The expression vector of the invention also contains a sequence which allows expression of hTOPI gene to be modulated (i.e., the *lac*I^q repressor). The expression vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, stability elements which provide mitotic stability to

5 the plasmid, and sequences which provide sites for cleavage by restriction endonucleases. The characteristics of the actual expression vector used must be compatible with the host cell which is to be employed.

[0012] The DNA coding for hTOP1 is well known in the art, for example, the sequence is disclosed in D'Arpa, P. et al. Proc. Natl. Sci. U.S.A. 85 p. 2543-2547, 1987 and Bjornsti, M.-A. et al., Cancer Reserach 49, p. 6318-6323, 1989.. The promoters useful herein are well known in the art and are chosen to be operable in the host cell of choice. Preferred

10 promoters include, for example, *lac, trp* and most preferred *tac*. The sequences for the promoters are described in, for example, Weiss et al. Proc. Natl. Acad. Sci. U.S.A. 81, p. 6019-6023, 1984.

[0013] The expression vector of the invention also preferably contains a DNA sequence coding for a selectable marker. The particular selectable marker used is not critical provided the marker allows for phenotypic selection in transformed host cells. For convenience, preferred selectable markers are antibiotic resistance. Examples of antibiotic

15 resistance selectable markers include ampicillin, tetracycline, kanamycin, chloramphenicol, spsctinomycin. Most preferred is streptomycin resistance, spectmomycin resistance or both.

[0014] The expression vector of the invention also contains *lac* I^q DNA which encodes a repressor. The repressor DNA sequence is disclosed in Farabaugh, Nature 274, p. 765-769, 1978.

[0015] Particularly preferred is the expression vector designated pMStopI, described in Figure 1, which contains the

20 DNA sequence coding for hTOPI, or expression vectors with the identifying characteristics of pMStopI.

[0016] Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques as taught herein or as known in the art, many of which are described in T. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

[0017] The present invention additionally concerns host cells containing an expression vector which comprises a

25 DNA sequence coding for all or part of hTOPI. The host cells preferably contain the pMStopI expression vector as shown in Figure 1. Further preferred are host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of and operatively linked to a DNA sequence coding for all or part of hTOPI. Suitable host cells are prokaryotic cells which are preferably biologically pure. Suitable prokaryotic host cells include, for example, *Escherichia coli, Bacillus subtilus* and *Salmonella typhimurum* cells. The

30 most preferred host cell of the invention is *E. coli* ATCC 69736. *E.coli* ATCC 69736 (also known as strain 2055A, RFM2055, or SBG 1398) was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A 20852 on December 13, 1994, under the provisions of the Budapest Treaty. *E. coli* ATCC 69736 contains plasmid pMStop1, a chromosomal *sulA-lac* insertion, and the chromsomal *imp* 4312 mutation.

[0018] Expression vectors may be introduced into host cells by various methods known in the art. For example,

35 transfection of host cells with expression vectors can be carried out by the calcium phosphate precipitation method. However, other methods for introducing expression vectors into host cells, for example, electroporation, biolistic fusion, liposomal fusion, nuclear injection, viral or phage infection or protoplast fusion, can also be employed.

[0019] Once an expression vector has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of the desired polypeptide, in this case a polypeptide molecule comprising all

40 or part of hTOPI.

[0020] Host cells containing an expression vector which contains a DNA sequence coding for all or part of hTOPI may be identified by one or more of the following six general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of marker gene functions; (c) assessing the level of transcription as measured by the production of hTOPI mRNA transcripts in the host cells; (d) detection of the gene product immunologically; (e) complementation analysis;

45 and (f) enzyme assay, enzyme assay being the preferred method of identification.

[0021] In the first approach, the presence of a DNA sequence coding for all or part of hTOPI can be detected by DNA-DNA or RNA-DNA hybridization using probes complementary to the DNA sequence.

[0022] In the second approach, the recombinant expression vector host system can be identified and selected based upon the presence of absence of certain marker gene functions (e.g., thymidine kinase activity, resistance to antibiotics,

50 uracil prototrophy, etc.). A marker gene can be placed in the same plasmid as the DNA sequence coding for all or part of hTOPI under the regulation of the same or a different promoter used to regulate the hTOPI coding sequence. Expression of the marker gene in response to induction or selection indicates expression of the DNA sequence coding for all or part of hTOPI.

[0023] In this third approach, the production of hTOPI mRNA transcripts can be assessed by hybridization assays.

55 For example, RNA can be isolated and analyzed by Northern blotting or nuclease protection assay using a probe complementary to the RNA sequence. Alternatively, the total nucleic acids of the host cell may be extracted and assayed or hybridization to such probes.

[0024] In the fourth approach, the expression of all or part of hTOPI can assessed immunologically, for example, by

Western blotting.

[0025] In the fifth approach, the expression of hTOPI protein can be assessed by complementation analysis. For example, in cells known to be deficient in this enzyme, expression of hTOPI activity can be inferred by improved growth of cells under growth-limiting conditions.

[0026] In the sixth approach, expression of hTOPI can be measured by assaying for hTOPI enzyme activity using known methods. For example, the assay described in Y. Pommier, J. Biol. Chem., 265, pages 9418-9422, 1990 may be employed.

[0027] The DNA sequences of expression vectors, plasmids or DNA molecules of the present invention may be determined by various methods known in the art. For example, the dideoxy chain termination method as described in Sanger et al., Proc. Natl. Acad. Sci. USA, 74, pages 5463-5467, 1977, or the Maxam-Gilbert method as described in Proc. Natl. Acad. Sci. USA, 74, pages 560-564, 1977 may be employed.

[0028] It should, for course, be understood that not all expression vectors and DNA regulatory sequences will function equally well to express the DNA sequences of the present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art may make a selection among expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

[0029] The present invention further concerns a method for detecting posion of hTOPI. The term "poison" means a compound which traps the cleavable-complex intermediate. The "cleavable-complex intermediate" is formed when an hTOPI poison traps the covalent phosphotyrosine DNA-protein intermediate of the strand passage reaction. A typical example of a poison is CMPT. .

[0030] Previously, it has been known that eTOPI enzyme is resistant to CMPT. We have also found that the bacterium, E. coli, is also resistant to CMPT. Prior art plasmids containing DNA coding for hTOPI are detrimental to bacteria, and E. coli in particular, and prior art plasmids are not stable. Such prior art plasmids are detrimental to bacteria because of topological problems resulting from an imbalance in DNA relaxing and DNA supercoiling activities. By use of the expression vector of the present invention, a lower copy number is achieved. Furthermore, the use of lacI^q gene with its repressor results in a more controllable expression system. The expression system of the invention is more controllable because the copy number of the hTOPI gene has been reduced by using a reduced copy number plasmid and the lacI^q gene has been provided on the same plasmid to insure elevated levels.

[0031] In the process of the invention, the host cell has an imp mutation, for example, the imp 4213 mutation. The imp mutation is described in B.A. Sampson et al., Genetics, 122, pages 491-501, 1989. We have found that using host cells with the imp mutation allows for entry of CMPT and similar compounds into the cells. As a result, bacteria such as E. coli can demonstrate sensitivity to CMPT and like drugs.

[0032] Moreover, in the process of the invention we note that DNA damage resulting from the addition of CMPT, if and only if hTOP is induced, can be detected through the induction of DNA damage. Inducible promoters such as sulA which is a part of the so-called SOS response can be used to detect such damage. We also infer that DNA damage has occurred in response to CMPT under the above defined conditions by the elevated cytotoxicity noted in a strain defective in DNA repair due to a loss of the recA gene.

[0033] In the process of the invention for detecting a poison to hTOPI, DNA damage is monitored. This can be accomplished by quantitating increases in the expression of the sulA gene. This can conveniently be performed by monitoring β-galactosidase production, for example, by monitoring a chromophor color change. Thus, in the method of the invention it is preferred that the host cell has a SOS responsive sulA-lac fusion.

[0034] The following examples are to illustrate the invention.

Example 1

E.coli expresses phenotypes based on the expression of hTOPI.

[0035] M.-A. Bjornsti et al., Cancer Research, 49, pages 6318-6323 (1989) have shown that the expression of an hTOP1 clone in E.coli can reverse the growth defect seen at 42°C in a topA amber mutant that was suppressed by a temperature sensitive amber suppressor. In a similar fashion we show the suppression of another phenotype associated with a defect resulting from the deletion of the topA gene.

[0036] It has been shown that a deletion of the topA gene cannot be inherited in a wild type E. coli but that certain defects in the gyrA or gyrB genes, which result in reduced DNA supercoiling, are able to allow the inheritance of a topA deletion (G. J. Pruss et al., Cell, 31, pages 35-42, 1982; S. DiNardo et al., Cell, 31, pages 43-51, 1982). The loss of the topA gene results in the loss of the cell's major DNA relaxing activity and, apparently, this loss is not viable unless balanced by a reduction in cell's capacity to supercoil DNA. The topA-deletion phenotype used herein is based on a strain in which a conditional defect in the gyrB gene is able to act as a conditional compensatory mutation for a topA deletion. The strain RFM475, which has both a temperature sensitive defect in the gyrB gene (gyrB221 gyrB203) and

a deletion in the chromosome that removes the *top*A and *cys*B genes (Δ*cystop*), is both temperature sensitive and cold sensitive. The temperature sensitive character of RFM475 is not unexpected as the *gyr*B gene product is temperature sensitive (R. Menzel et al., Cell, 34, pages 105-113, 1983). The cold sensitive phenotype is novel. At 37°C the mutant gyrase allele is defective but retains enough activity to allow the strain to grow, and this reduced activity allows the

5 inheritance and stable maintenance of the *top*A deletion. Lowering the temperature of the strain to 25°C results in the mutant gyrase regaining a more wild type level of activity and the strain is unable to grow at 25°C.

[0037] The ability to observe transformants at 25°C in RFM475 (Δ*top*A *gyr*B221 *gyr*B203) is used herein as a phenotypic means of demonstrating that a plasmid carries DNA relaxing activity. Table I shows that the plasmid pUC19 is able to transform the strain RFM475 to ampicillin (AP) resistance only at 37°C, whereas, in the strain RFM445 (*gyr*B221

10 *gyr*B203 *top*A⁺) AP resistant transformants are obtained at both 25°C and 37°C. Using a control plasmid (pID35; K. Becherer et al., Nucl. Adds Res., 11, pages 1773-1790, 1983; L. Zumstein et al., J. Mol. Biol., 191, pages 333-340, 1986) which contains a truncated, but active, form of the *E. coli top*A gene transformants are obtained in both RFM475 and RFM445 at 25°C and 37°C. The truncated version of the *E. coli top*A gene is able to suppress the cold sensitive defect of RFM475. (The truncated version of *top*A is used because full length *top*A clones on a high copy number

15 plasmid show stability problems). The hTOPI cDNA clone (obtained from Jim Wang; M.A. Bjornsti et al., Proc. Natl. Acad. Sci. USA, 84, pages 8971-8975, 1987) is able to give AP-resistant transformants at 25°C in the *gyr*B^{TS} Δ*top*A strain. In addition it is noted that it is not able to observe transformants with the *gyr*B^{TS} *top*A⁺ strain using the hTOPI cDNA clone when the transformation mixture is plated at 37°C while normal transformation is noted at 25°C in this genetic background. Apparently the addition of more DNA relaxing activity to a strain with a defect in DNA supercoiling,

20 in the case of the hTOPI clone, can extend the lethal range of the *gyr*B^{TS} gyrase allele to 37°C. It is worth noting that while the eTOPI clone (pID35) is able to suppress the cold sensitivity of RFM475, it does not extend the temperature sensitivity of the *gyr*B^{TS} allele to 37°C. Table 1 shows two phenotypes consistent with the hTOPI cDNA clone demonstrating DNA relaxing activity in *E. coli*: the ability to suppress the cold sensitivity associated with the loss of *top*A and the potential to extend the lethal range of a *gyr*B^{TS} allele.

25 [0038] The reason for using the ability to obtain transformants in a particular genetic background to demonstrate that a gene carried on a plasmid has certain phenotypes, in this instance, associated with DNA topology lies in the unstable character of the transformants that are obtained with the ptac-hTOP plasmid.

Table 1

Host Strain	Plasmid	Transformants		
		@25°C	@37°C	@42°C
RFM443 gyrB+ topA+	pUC19	YES	YES	YES
	pID35 (eTOPI)	YES	YES	YES
	ptac-hTOPI	YES*	YES*	YES*
RFM445 gyrB^TS topA+	pUC19	YES	YES	NO
	pID35 (eTOPI)	YES	YES	NO
	ptac-hTOPI	NO	NO	NO
RFM475 gyrB^TS Δtop	pUC19	YES*	NO	NO
	pID35 (eTOPI)	YES	YES	NO
	ptac-hTOPI	YES*	YES*	NO

The plasmid ptac-hTOP can demonstrate phenotypes consistent with the introduction of DNA relaxing activity. The indicated strains were transformed with either pUC19 (no insert), pID35 (a clone with a truncated, but active, version the E.coli topA gene extending from the Pstl site upstream of the topA gene to the EcoRI site within the carboxyl domain of the topA gene cloned into pBR322, K. Becherer et al., Nucl. Acids Res. 11, pages 1773-1790, 1983; L. Zumstein et al., J. Mol. Biol., 191, pages 333-340, 1986), or ptac-hTOP (a full length cDNA clone of the gene for hTOPI expressed from a tac promoter, M.-A. Bjornlsi et al., Cancer Research, 49, pages 6318-6323, 1989) on LB-agar plates (T. J. Silhavy et al., Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) with 50 μgram/ml AP. All strains were made competent using standard CaCl$_2$ methods (J. Sambrook et al., Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) and 2 X 10^{8} cells were transformed with 10 nanograms of CsCl₂ banded plamsid DNA (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). After a 5 minute heat shock at 37°C, a 0.1X aliquot of the mixture was plated at the indicated temperatures. Plates were score for the inheritance of the plasmid (AP^R) at the indicated temperatures after 16 hours for the 37°C and 42°C plates, and after 24 hours for the 25°C plates. A "YES" in the table indicates that 100-1000 uniform smooth rounded colonies with modest colony size variation appeared. A "YES*" indicates that 100-1000 transformants with irregular colony morphology and vastly different sizes showing frequent fast growing papillae arose. A "NO" indicates that transformants were not observed following the designated incubation times.

[0039]   **Host strains.** The gyrB+ topA+ recipient is RFM443 which is a Δlac74 version of the E.coli wild type N99 or W3102 (Bachman, B. J., Derivation and genotypes of some mutant derivatives of Escherichia coli K-12. pages 1190-1220 in, Escherichia coli and Salmonella typhimurium cellular and molecular biology. Chief Editor, F.C. Neidhardt, ASM Press 1987). The gyrB^{TS} topA+ recipient is RFM445, a Δlac74 version of N4157 (an N99/W3102 derivative) containing the defective gyrase allele (gyrB221, gyrB203) previously described (R. Menzel et al., Cell, 34, pages 105-113, 1983).

[0040]   The strain RFM475 was constructed as follows: N4157 was transduced with phage P1vir grown on PLK831 to pyrF ΔtrpE zci-Tn10 by for selecting tetracycline resistance. An appropriate transductant from that cross was transduced to Pyr+ with phage grown on N99 and a ΔtrpE-tetracycline sensitive transductant was retained as RFM431. RFM431 was transduced to Trp+ with phage grown on DM800 (ΔcysBtopA, S. DiNardo et al., Cell, 31, pages 43-51, 1982) and a Cys- (ΔcysBtopA) transductant was made Δlac74 as described below and kept as RFM475. (RFM431, 443, 445, and 475 are also described in M. Drolet et al., Proc. Natl. Acad. Sd. USA, 1995 in press).

Reduced hTOPI expression leads to more stable strains.

[0041]   Expression of the hTOPI gene in the plasmid ptac-hTOP is directed by the strong synthetic tac promoter. This promoter is subject to regulation by the lacI gene (H. A. deBoer et al., Proc. Natl. Acad. Sci. USA, 80, pages 21-25, 1983, but when present on a high copy number plasmid a single chromosomal copy of the lacI gene is not sufficient to repress expression. Extra copies of the lacI gene on second plasmids (F'lac I⁹ and pMS421) were provided and copy of the over-expressing lac I⁹ gene was cloned onto the plasmid ptac-hTOP. Neither of these approaches would yield stable transformants. Apparently the basal repressed expression of the hTOPI gene from the ptac-hTOPI construct is not well tolerated in a wild type E. coli. To reduce this basal expression the hTOPI gene from ptac-hTOP was into the subcloned lower copy number plasmid pMS421 (pMS421 has a pSC101-origin of replication and the lac I⁹ gene; D. Graña et al., Genetics, 120, pages 319-327, 1988) as illustrated in Figure 1. In the absence of the tac inducer, IPTG, transformants of our subclone, pMStopI, are uniform in colony size and morphology, and appear to be completely stable. Upon the addition of IPTG (0.1 - 1 mM) the complementation patterns and plasmid instability discussed above reappear. Recloning the gene from a pUC-based to a pSC101- based plasmid, together with the introduction of a tighter repression control, allows for the stable repressed inheritance of the hTOPI gene in E.coli, while permitting the IPTG induced expression of phenotypes when required.

Example 3

A strain with PMStopI can be made to show an inducible camptothecin sensitivity.

[0042]   The eukaryotic type I topoisomerases are unique in their ability to relax DNA in the absence of a divalent cation and the inclusion of a chelating agent, such as EDTA, assures that only eukaryotic topoisomerase I is being assayed (P.-H. Vosberg, Curr. Top. Microbiol. Immunol., 114, pages 19-102, 1985; M.-A. Bjornsti et al., Proc. Natl. Acad. Sci. USA, 84, pages 8971-8975, 1987). Crude lysates prepared from cells harboring pMStopI grown in the presence of 1 mM IPTG demonstrate abundant EDTA-resistant DNA relaxing activity, while the same cells grown without IPTG fail to demonstrate (<5% induced levels) such activity (data not shown). Furthermore, the EDTA resistant DNA relaxing activity expressed upon IPTG induction is inhibited by the anti-cancer drug CMPT. CMPT inhibits hTOPI by trapping an enzyme-DNA intermediate in the so-called cleavable complex (Y.-H. Hsiang et al., J. Biol. Chem., 260, pages 14873-14878, 1985; and E. Kjeldsen et al., Characterization of a camptothecin-resistant human DNA topoisomerase I., J. Biol. Chem., 263, pages 3912-3916, 1988). In vitro the enzyme-DNA intermediate trapped in such a cleavable complex can be provoked to demonstrate single stranded breaks by the addition of a protein denaturant such as SDS (Y.-H. Hsiang et al., J. Biol. Chem., 260, pages 14873-14878, 1985). In vivo, activities in eukaryotic cells, such as DNA replication, process the cleavable complex into extensive DNA damage which results in cell death (L. Liu, Annu. Rev. Biochem., 58, pages 351-375, 1989). Since bacteria harboring pMStopI grown in the presence of IPTG contain a CMPT sensitive DNA relaxing activity, it was reasoned that such cells should be sensitive to the effects of

the CMPT. Initial experiments failed to demonstrate any CMPT sensitivity for cells harboring pMStopl grown in the presence of 1mM IPTG (see Table 2).

[0043] There could be many reasons for the hTOPI-expressing bacteria failing to demonstrate CMPT sensitivity. One such reason could be lack of permeability to CMPT. Several genetic backgrounds and mutations were tried in an effort to demonstrate an inducible CMPT sensitivity with the pMStopl plasmid. The highest level of sensitivity was found when the *imp*4213 mutation was introduced. The *imp* mutant was originally isolated by selecting for increased permeability to maltodextran and was subsequently shown to have dramatically increased sensitivity to several drugs and chemical agents (B. A. Sampson et al., Genetics, 122, pages 491-501, 1989). The *imp* mutation was mapped to minute 2 on the *E.coli* map but the biochemical nature of the lesion is not known (B. A. Sampson et al., Genetics, 122, pages 491-501, 1989; Benson, personal communication). In Table 2 the combination of the *imp*4213 mutation, the plasmid pMStopl, and growth with IPTG are shown to be all necessary for a CMPT sensitive *E.coli*.

Table 2

Strain	*imp+ /pMStopl*		*imp4213 /pMS421*		*imp4213 /pMStopl*	
IPTG	None	+1mM	None	+1mM	None	+1mM
Camptothecin 200 µg/ml						
50	4	4	7	7	7	15
25	4	4	7	7	7	13
12	4	4	7	7	7	11
6.2	4	4	7	7	7	9
None	4	4	7	7	7	7

hTOPI expression and an *imp* defect are required for CMPT sensitivity. 10 cm petri dishes with 40 ml of LB-agar plus 50 µgram/ml AMP, with (1mM) or without IPTG, were overlayed with 10^8 midlog cells of the designated genotype in 4ml of soft (0.7% agar) LB-agar at 48°C. When the soft-agar overlays had solidified, 4mm cylindrical holes were cored to the bottom surface of the petri dish using a small probe attached to a vacuum. 20ul samples of CMPT (Sigma) dissolved in DMSO at the concentrations indicated were applied to these wells; "None" indicates a DMSO-alone control. After 16 hours at 30°C, the zones of clearing (no cell growth) around the wells were measured and are recorded as millimeters of diameter in the table. The 4 mm value for the *imp+*/pMStop1 strain is simply the diameter of the well. The 7mm value noted for both *imp*4213 strains in the "None" row, DMSO-alone control, shows that the *imp* defect confers a low level of sensitivity to 100% DMSO.

[0044] **Strains used.** The *imp+*/pMStop1 strain is N99 transformed with pMStop1. An *imp*4213 derivative of *E. coli* strain N99 was constructed by growing P1*vir* on BAS1329 (*imp*4213 *leu*::Tn *10*, B. A. Sampson et al., Genetics, 122, pages 491-501, 1989) and tranducing N99 to tetracycline resistance and screening for coinheritence of the *imp* phenotype as described in Sampson *at al.* 1989. Such an *imp-* strain was then transduced to protrophy (Leu+) and an *imp*4213 version was retained as RFM795. The strains *imp*4213/pMS421 and *imp*4213/pMStop1 are RFM795 transformed with pMS421 and pMStop1 respectively (on LB-agar selecting SPC, resistance at 25 µgram/ml).

Example 4

Camptothecin sensitivity in *E.coli* Involves DNA damage.

[0045] The pathway leading from the formation of the CMPT-induced cleavable complex to cell death involves DNA damage (L. Liu, Annu. Rev. Biochem., 58, pages 351-375, 1989). This has been demonstrated in the yeast *S. cerevisiae* through the elevated CMPT sensitivity seen in a DNA damage repair deficient *rad*52 mutant and through the induction of a DNA damage inducible promoter following exposure to CMPT (J. Nitiss et al., Proc. Natl. Acad. Sci. USA, 85, pages 7501-7505, 1988; W.-W. Eng et al., Mol. Pharm., 34, pages 755-760, 1989). We are able to make similar observations in *E.coli* strains harboring the pMStopl plasmid.

[0046] In *E.coli* the activation of the *rec*A gene product and the subsequent inactivation of the *lex*A repressor are primary events in the induction of many enzymes involved in the repair of DNA damage (J. W. Little et al., Cell, 29, pages 11-22, 1982). Mutants defective in *rec*A consequently exhibit enhance sensitivity to many DNA damaging agents (G. A. Walker, Microbiol. Rev. 48, pages 60-93, 1984). In Table 3 it is demonstrated that an *E.coli* strain with a defect in the *rec*A gene shows 50-fold enhanced sensitivity to CMPT when the expression of hTOPI is induced. Presumably with a *rec*A defect the repair of CMPT-induced damage does not occur and such a strain is, therefore, more sensitive (than a *rec*A+ strain) to the effects of this antitumor drug when hTOPI is present.

[0047] The *sul*A gene product is able to inhibit cell division and its induction following DNA damage is thought to allow the cell sufficient time for DNA repair to occur prior to the next cell division (Huisman and D'Ari, 1982). Measuring increases in β-galactosidase activity directed by a *sul*A-*lac* gene fusion is an established method for monitoring DNA damage (Kenyon et al., 1982). In Figure 2A we show that CMPT is able to induce *sul*A only when hTOPI is present. This behavior stands in contrast to other DNA damaging agents, such as nalidixic acid (Figure 2B), which do not depend on the expression of hTOPI. The hTOPI dependence of a number of known DNA damaging agents was examined and only CMPT has a signal which is dependent upon the induction of hTOPI (data not shown). Most DNA damaging agents show a reduced capacity (0.5X to 0.9X) to induce *sul*A-*lac* when hTOPI is expressed (data not shown), and the basal (uninduced) level of *sul*A expression declines 20% upon hTOPI induction. This is a result of the fact that hTOPI expression is detrimental to *E.coli* and less robust *E.coli* are less able to express or increase expression of *sul*A.

Table 3

Strain	rec A+		rec A−	
IPTG	None	1mM	None	1mM
Camptothecin µg/ml				
25	7	11	7	>20
10	7	7	7	17
5	7	7	7	16
2.5	7	7	7	14
1.2	7	7	7	13
0.63	7	7	7	11
0.31	7	7	7	9
None	7	7	7	7

[0048] A *rec*A defect elevates CMPT sensitivity. The strain RFM795 (as described for Table 2) was transduced to tetracycline resistance with P1*vir* grown on a strain with the Δ*srlrec*A *srl*::dTn *10* mutation (a deletion of *rec*A generated from a Tn*10* in *srl*) to obtain a *rec*A defective version of RFM795. Both Rec- and Rec+ versions were transformed with pMStop1 (selected on 25 µgram/ml SPC LB-agar) to obtain the strains used. Plates, agar overlays, and sample application was performed as described for Table 2. The results show that 0.63 µgrams/ml CMPT gives the same IPTG-dependent zone of inhibition in the *rec*A defective strain as noted at 25 µgrams/ml in the *rec*A+ strain.

Claims

1. An expression vector comprising:

    (a) human topoisomerase I (hTOPI) DNA,
    (b) a promoter capable of directing expression of hTOPI, and
    (c) lacI^q DNA which encodes a repressor for the promoter of (b).

2. The expression vector of Claim 1 wherein the hTOPI DNA is cDNA.

3. The expression vector of Claim 1 further comprising an origin of replication and a DNA sequence which encodes a selectable marker.

4. The expression vector of Claim 3 wherein the selectable marker is antibiotic resistance.

5. The expression vector of Claim 3 wherein the selectable marker is streptomycin resistance, spectmomycin resistance or both.

6. The expression vector of Claim 1 wherein the promoter is *lac, trp* or *tac.*

7. The expression vector of Claim 1 having the identifying characteristics of plasmid pMStopI, as deposited with ATCC and having the accession number ATCC 69736.

8. A prokaryotic host cell containing an expression vector as claimed in anyone of claims 1 to 7.

9. The prokaryotic host cell of claim 8 which is *E. coli.*

10. The *E. coli* host cell of Claim 9 which contains an *imp* mutation.

11. The *E. coli* host cell of Claim 10 wherein the *imp* mutation is *imp* 4213 mutation.

12. *E. coli* ATCC 69736.

13. A method for detecting a poison of hTOPI, which poison is capable of trapping the cleavable complex-intermediate as formed by hTOPI and a DNA molecule, comprising:

   (A) culturing a prokaryotic host cell containing the *imp* mutation and an expression vector of claim 1 containing hTOPI DNA under conditions sufficient to express human topoisomerase I, in the presence of a sample which may contain a human topoisomerase I poison, and
   (B) monitoring DNA damage of the host cell.

14. The method of Claim 13 wherein the prokaryotic host cell is *E. coli.*

15. The method of Claim 13 wherein the expression vector further comprises an origin of replication and a DNA sequence which encodes a selectable marker.

16. The method of Claim 15 wherein the hTOPI DNA is cDNA, the selectable marker is antibiotic resistance to streptomycin, spectmomycin, or both, and the promoter is *lac, trp,* or *tac.*

17. The method of Claim 13 wherein the expression vector has the identifying characteristics of plasmid pMStopI as deposited with ATCC and having the accession number ATCC 69736.

18. The method of Claim 14 wherein the *E. coli* has an SOS responsive *sulA-lac* fusion.

19. The method of Claim 18 wherein the monotoring of DNA damage is performed by monitoring β-galactosidase production.

20. The method of Claim 19 wherein β-galactosidase production is monitored by chromophor color change.

21. A method for detecting a poison of hTOPI, which poison is capable of trapping the cleavable complex-intermediate as formed by hTOPI and a DNA-molecule, comprising

   (A) culturing an *E. coli* host cell containing the *imp* mutation and plasmid pMStopI as deposited with ATCC and having the accession number ATCC 69736 in the presence of a sample which may contain a human topoisomerase I poison, and
   (B) monotoring β-galactosidase production by chromophor color change.

22. The method of one of the claims 13 to 21, wherein the poison is camptothecin.

Patentansprüche

1. Expressionsvektor umfassend:

   (a) humane Topoisomerase I (hTOPI) DNA,
   (b) einen Promotor, der zur Steuerung der Expression von hTOPI befähigt ist, und
   (c) lacIq DNA, welche für einen Repressor des Promotors gemäß (b) codiert.

2. Expressionsvektor nach Anspruch 1, worin die hTOPI DNA eine cDNA ist.

3. Expressionsvektor nach Anspruch 1, der zusätzlich einen Replikationsursprung und eine für einen selektierbaren Marker codierende DNA-Sequenz umfaßt.

4. Expressionsvektor nach Anspruch 3, worin der selektierbare Marker eine Antibiotikaresistenz ist.

5. Expressionsvektor nach Anspruch 3, worin der selektierbare Marker eine Streptomycin-Resistenz, eine Spectmo-mycin-Resistenz oder beides ist.

6. Expressionsvektor nach Anspruch 1, worin der Promotor ein lac-, trp- oder tac-Promotor ist.

7. Expressionsvektor nach Anspruch 1 mit den identifizierenden Merkmalen von Plasmid pMStopI, hinterlegt bei der ATCC under der Hinterlegungsnummer ATCC 69736.

8. Prokaryotische Wirtszelle, enthaltend einen Expressionsvektor nach einem der Ansprüche 1 bis 7.

9. Prokaryotische Wirtszelle nach Anspruch 8, nämlich E. coli.

10. E. coli-Wirtszelle nach Anspruch 9, welche eine imp-Mutation enthält.

11. E. coli-Wirtszelle nach Anspruch 10, worin die imp-Mutation die imp 4213-Mutation ist.

12. E. coli ATCC 69736.

13. Verfahren zum Nachweis eines hTOPI-Giftes, welches dazu befähigt ist, den durch hTOPI und einem DNA-Molekül gebildeten spaltbaren intermediären Komplex einzufangen, wobei man:

    (A) eine prokarotyische Wirtszelle, welche die imp-Mutation und einen die hTOPI-DNA enthaltenden Expressionsvektor nach Anspruch 1 enthält, unter Bedingungen, die zur Expression von humaner Topoisomerase I geeignet sind, in Gegenwart einer Probe kultiviert, welche das Gift gegen humane Topoisomerase I enthalten kann, und (B) die DNA-Schädigung der Wirtszelle bestimmt.

14. Verfahren nach Anspruch 13, worin die prokaryotische Wirtszelle E. coli ist.

15. Verfahren nach Anspruch 13, worin der Expressionsvektor außerdem einen Replikationsursprung und eine für einen selektierbaren Marker codierende DNA-Sequenz umfaßt.

16. Verfahren nach Anspruch 15, worin die hTOPI-DNA eine cDNA ist, der selektierbare Marker eine Resistenz gegenüber Streptomycin, Spectmomycin oder gegenüber beide ist und der Promotor ein lac-, trp- oder tac-Promotor ist.

17. Verfahren nach Anspruch 13, worin der Expressionsvektor die charakteristischen Merkmale des Plasmids pMStopI, hinterlegt bei der ATCC unter der Hinterlegungsnummer ATCC 69736 aufweist.

18. Verfahren nach Anspruch 14, worin E. coli eine SOS-ansprechende sulA-lac-Verknüpfung aufweist.

19. Verfahren nach Anspruch 18, worin die Bestimmung der DNA-Schädigung über die Bestimmung der β-Galaktosi-dase-Produktion erfolgt.

20. Verfahren nach Anspruch 19, worin die β-Galaktosidase-Produktion durch den Farbwechsel eines Chromophors bestimmt wird.

21. Verfahren zum Nachweis eines hTOPI-Giftes, welches dazu befähigt ist, den durch hTOPI und einem DNA-Molekül gebildeten spaltbaren intermediären Komplex einzufangen, wobei man:

(A) eine E. coli-Wirtszelle, welche die imp-Mutation und das Plasmid pMStopI, hinterlegt bei der ATCC unter der Hinterlegungsnummer ATCC 69736 enthält, in Gegenwart einer Probe kultiviert, welche das Gift für humane Topoisomerase I enthalten kann, und
(B) über den Farbwechsel eines Chromophors die β-Galaktosidase-Produktion bestimmt.

22. Verfahren nach einem der Ansprüche 13 bis 21, worin das Gift Camptothecin ist.

Revendications

1. Vecteur d'expression comprenant :

   (a) l'ADN codant pour la topo-isomérase humaine (hTOPI), (b) un promoteur capable de diriger l'expression de hTOPI, et
   (c) l'ADN lacI^q qui code pour un répresseur du promoteur de (b).

2. Vecteur d'expression de la revendication 1, dans lequel l'ADN de hTOPI est de l'ADNc.

3. Vecteur d'expression de la revendication 1, comprenant en outre une origine de réplication et une séquence d'ADN qui code pour un marqueur sélectionnable.

4. Vecteur d'expression de la revendication 3, dans lequel le marqueur sélectionnable est une résistance à un antibiotique.

5. Vecteur d'expression de la revendication 3, dans lequel le marqueur sélectionnable est une résistance à la streptomycine, une résistance à la spectmomycine ou les deux.

6. Vecteur d'expression de la revendication 1, dans lequel le promoteur est *lac, trp* ou *tac.*

7. Vecteur d'expression de la revendication 1, ayant les caractéristiques d'identification du plasmide pMStopI, tel qu'il est déposé à l'ATCC et ayant le numéro d'accès ATCC 69736.

8. Cellule hôte procaryote contenant un vecteur d'expression tel que revendiqué dans l'une quelconque des revendications 1 à 7.

9. Cellule hôte procaryote de la revendication 8, qui est *E. coli.*

10. Cellule hôte *d'E. coli* de la revendication 9, qui contient une mutation *imp.*

11. Cellule hôte d'*E. coli* de la revendication 10, dans laquelle la mutation *imp* est la mutation imp 4213.

12. *E. coli* ATCC 69736.

13. Méthode de détection d'un poison de hTOPI, ce poison étant capable de piéger le complexe intermédiaire pouvant être coupé, qui est formé par hTOPI et une molécule d'ADN, comprenant :

    (A) la culture d'une cellule hôte procaryote contenant la mutation imp et un vecteur d'expression de la revendication 1 contenant l'ADN de hTOPI dans des conditions suffisantes pour l'expression de la topo-isomérase humaine I, en présence d'un échantillon qui peut contenir un poison de la topo-isomérase I humaine, et
    (B) le suivi des dommages subis par l'ADN de la cellule hôte.

14. Méthode de la revendication 13, dans laquelle la cellule hôte procaryote est *E. coli.*

15. Méthode de la revendication 13, dans laquelle le vecteur d'expression comprend en outre une origine de réplication et une séquence d'ADN qui code pour un marqueur sélectionnable.

16. Méthode de la revendication 15, dans laquelle l'ADN de hTOPI est de l'ADNc, le marqueur sélectionnable est une résistance à un antibiotique streptomycine, spectmomycine ou aux deux et le promoteur est *lac, trp* ou *tac.*

17. Méthode de la revendication 13, dans laquelle le vecteur d'expression a les caractéristiques d'identification du plasmide pMStopI, tel qu'il est déposé à l'ATCC et ayant le numéro d'accès ATCC 69736.

18. Méthode de la revendication 14, dans laquelle *E. coli* comprend une fusion *sulA-lac* qui répond à SOS.

19. Méthode de la revendication 18, dans laquelle le suivi des dommages subis par l'ADN est effectué en suivant la production de β-galactosidase.

20. Méthode de la revendication 19, dans laquelle la production de β-galactosidase est suivie par le changement de couleur d'un chromophore.
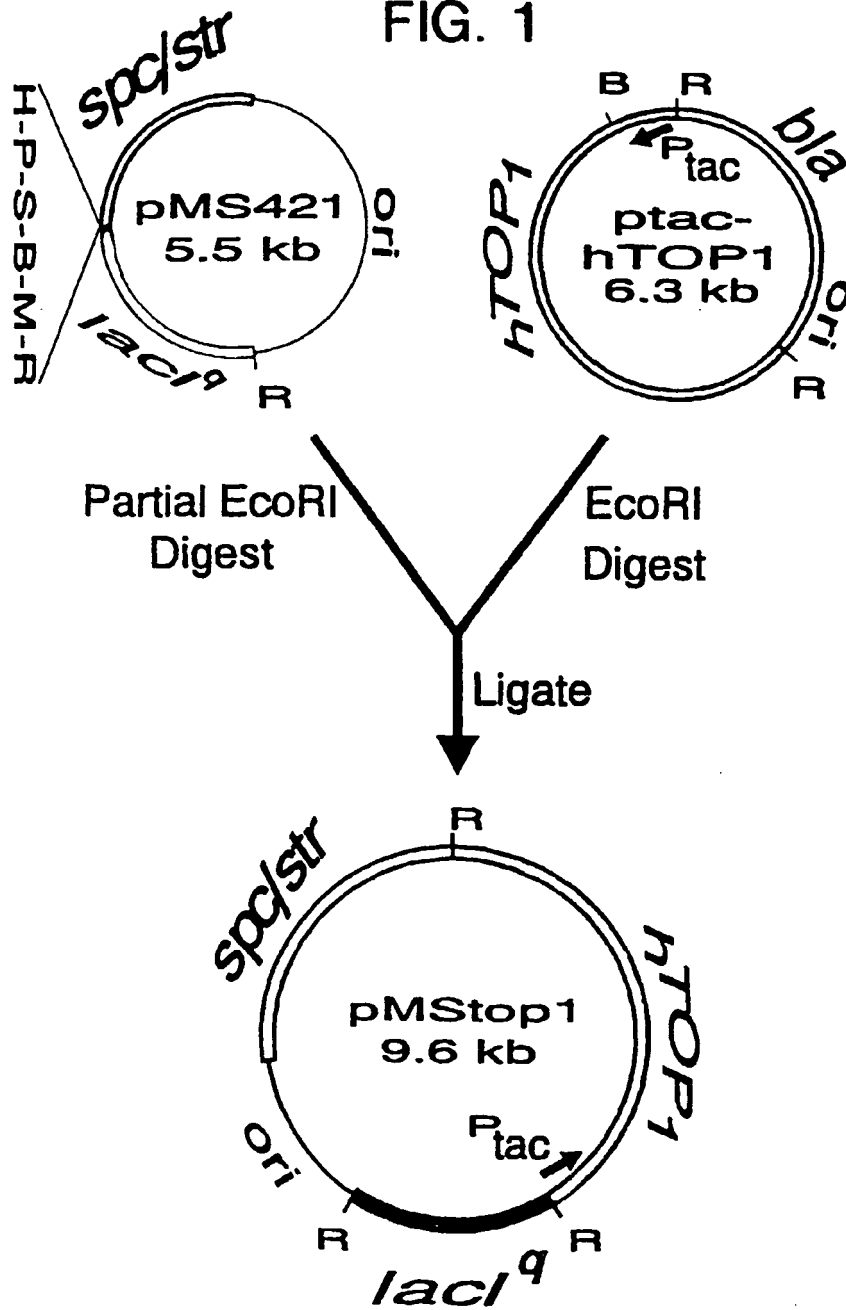
21. Méthode de détection d'un poison de hTOPI, ce poison étant capable de piéger le complexe intermédiaire pouvant être coupé qui est formé par hTOPI et une molécule d'ADN, comprenant :

(A) la culture d'une cellule hôte d'*E coli.* contenant la mutation *imp* et le plasmide pMStopI, tel qu'il est déposé à l'ATCC et ayant le numéro d'accès ATCC 69736 en présence d'un échantillon qui peut contenir un poison de la topo-isomérase I humaine, et
(B) le suivi de la production de β-galactosidase par le changement de couleur d'un chromophore.

22. Méthode de l'une quelconque des revendications 13 à 21, dans laquelle le poison est la camptothécine.

FIG. 1

(R)EcoRI (H)HinDIII (P)PstI (S)SalI (B)BamHI (M)SmaI
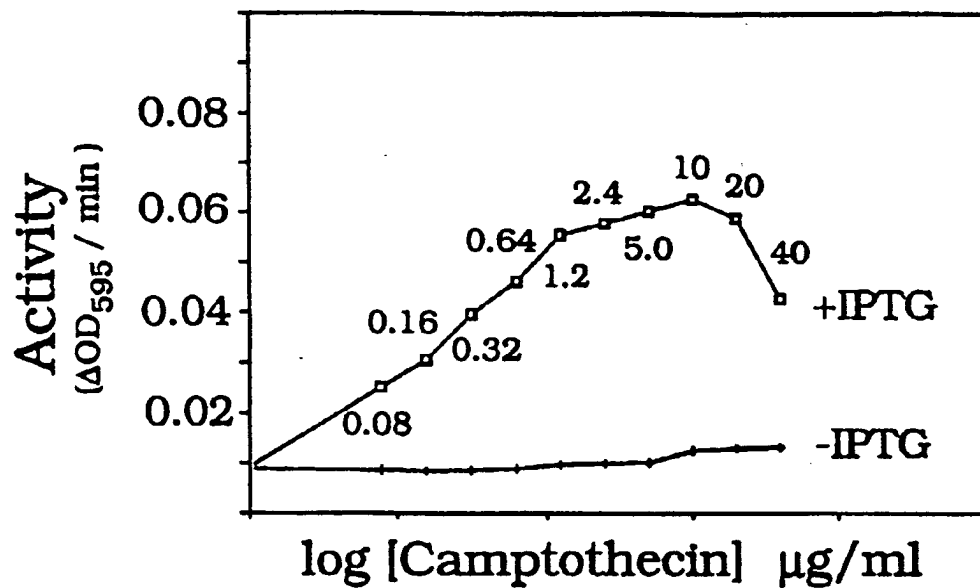
FIG. 2A
Activation of *sul*A- *lac*

FIG. 2B
Activation of *sul*A- *lac*